Mat. Res. Soc. Symp. Proc. Vol. 724 © 2002 Materials Research Society N6.4

Effects of Ionic Flow and Amelogenins on the Lengthwise Growth of Octacalcium Phosphate Crystals in a Model System of Tooth Enamel Formation.

M. Iijima[1], Y. Moriwaki[1], H.B. Wen[2#], T. Takagi[3], A.G. Fincham[2] and J. Moradian-Oldak[2]
[1] Asahi University School of Dentistry, Dental Materials and Technology, 1851-1 Hozumi, Hozumi-cho, Motosu-gun, Gifu 501-0296, Japan. [2] University of Southern California, School of Dentistry, Center for Craniofacial Molecular Biology, USA. # Present Affiliation : DePuy, a Johnson & Johnson company. [3] Tokyo Medical and Dental University, School of Dentistry, Oral Biology, Japan.

ABSTRACT

This paper briefly reviews our recent studies, which aimed to investigate the effects of 1) the Ca^{2+} and PO_4^{3-} ions flow and 2) amelogenins on the lengthwise growth of octacalcium phosphate (OCP), which is a potent precursor of enamel apatite crystal. OCP crystals were grown at 37°C in a dual membrane system under various amount of ionic inflow into a reaction space, using 1) 5-30mM Ca and PO_4 solutions as ionic sources and 2) extracted bovine amelogenin and recombinant murine amelogenins (rM179, rM166). With an increase in the amount of Ca^{2+} and/or PO_4^{3-} ions flow, the length of OCP crystal increased, while the width decreased. As a result, the length to width (L/W) ratio of crystal changed from 3 to 95, while the width to thickness (W/T) ratio from 32 to 9. The effect of amelogenins was unique, regardless of the type of amelogenins : Rod-like and prism-like OCP crystals with large L/W (61~107) and small W/T (1.3~2.2) ratios were formed in 10% amelogenin gels. In contrast, characteristic ribbon-like OCP crystals grew without protein and with gelatin, albumin, polyacrylamide gel and agarose gel. Specific interaction of amelogenins with OCP crystal was ascribed to the self-assembly property of amelogenin molecules and their hydrophobic nature. It was suggested that ionic flow and amelogenins play some critical roles in the elongated growth of enamel crystals.

INTRODUCTION

Enamel crystals of mammalian tooth are formed in an enamel matrix, which is abundant in amelogenins, under regulated Ca^{2+} and PO_4^{3-} ion supply from the layer of ameloblasts. In the early stage of the enamel crystal formation, very long and thin crystallites deposit in an enamel matrix with their long axis parallel to each other. In the later stage, crystals mainly increase their width and thickness, and grow into flat-hexagonal prisms [1,2]. The morphology is quite different from the irregular shaped plate-like morphology of bone and dentin crystals. We speculate that the uniqueness of enamel crystals relates to their growth condition : 1) lattice ions of enamel crystals, Ca^{2+} and PO_4^{3-} ions, are transported from the layer of ameloblasts into the enamel matrix, which might cause ionic flow, and the mode of the ionic flow changes during the tooth enamel formation [3,4] ; 2) molecules of amelogenin, which is major component of enamel proteins [5,6] and highly hydrophobic [7], assemble into nanospheres and form gel [8-10] with unique property [11].

We have been studying the mechanism of the lengthwise and oriented growth of enamel crystals based on a hypothesis that 1) octacalcium phosphate (OCP)-like phase is initiated as a precursor of enamel apatite; 2) one directional supply of the lattice ions contribute to the lengthwise growth in the c-axis direction ; 3) amelogenin nanospheres play roles as a scaffolding matrix in the highly organized growth of enamel crystals [reviewed in 12,13]. To evaluate the hypothesis, OCP crystals were grown in a model system of tooth enamel formation, where Ca^{2+} and PO_4^{3-} ions were supplied through membranes into amelogenin gels (a dual membrane system [14-16]). The present paper shows how ionic flow and amelogenins affected the growth of OCP crystals.

EXPERIMENTAL

A dual membrane system

Reactions were carried out at 37 °C and pH6.5 for 3days in a dual membrane system [14], where a cation selective membrane (CMV™) (Asahi Glass Co.) and a dialysis membrane (Visking Cellulose Tubing ; Union Carbide Co.) were used to control diffusion of Ca^{2+} and PO_4^{3-} ions. Membranes (about 8mm in diameter) were

attached to a Ca solution ($Ca(CH_3COO)_2 \cdot H_2O$, 1.8ml) container. The Ca solution container was put into a PO_4 solution ($NH_4H_2PO_4 + (NH_4)_2HPO_4$; 1:1 molar ratio, 100ml). Ca^{2+} and PO_4^{3-} ions diffuse into the reaction space between the membranes (about 15µl of volume) from mutually opposite sides respectively through the CMV and the dialysis membrane. Crystals deposited on the CMV or on the both membranes depending on the solution concentration.

Effect of ionic flow

To evaluate the effect of ionic flow on the growth mode, crystal growth was carried out under different amount of PO_4^{3-} and Ca^{2+} ions' flux [15]. One of factors that determine the driving force of diffusion of ions through a membrane is the potential difference across it. Therefore, amount of PO_4^{3-} and Ca^{2+} ions' influx across the membrane was changed by changing concentration of phosphate solution and Ca solution used as PO_4^{3-} and Ca^{2+} ionic sources. 5, 10, or 30mM of PO_4 and Ca solutions were used in different combinations.

Effects of amelogenins

To evaluate the effect of amelogenins, crystal growth was carried out in 10% amelogenin gels, using 10mM Ca and PO_4 solutions. Three types of amelogenins were used : 1) extracted bovine amelogenin and 2) recombinant murine amelogenins, rM179 (M=20kDa) and rM166 (M=16.8kDa). About 40% of the bovine amelogenin was 20.7kDa fraction, which lack the hydrophilic C-terminal residues of the full length amelogenin [17,18], and the rest was degraded fractions with the molecular weight of about 3-8, 13, 16kDa [14]. rM179 has the hydrophilic C-terminal residues and lacks an N-terminal methionine and a phosphorylated serine residue : rM166 lacks the hydrophilic C-terminal residues present in rM179 [19]. Their purity was higher than 95% [16]. 10% amelogenin solution was put in the reaction space and 10mM of Ca and PO_4 solutions were used as ionic sources. Some parallel reactions were carried out in 10% bovine serum albumin, gelatin, polyacrylamide (PAA) gel and 1% agarose gel for a comparison.

After a reaction, the precipitates still fixed on the membrane were rinsed superficially with distilled water and air dried or lyophilized when organic materials were used. Crystals were identified by an X-ray diffractometer (XRD) (Rigaku, RINT 2500). Morphology of crystals was observed by a scanning electron microscope (SEM) (Hitachi, S4500). Crystal size was measured on the SEM photographs, and their averages and standard deviations were calculated.

RESULTS

Effect of ionic flow

Morphology of OCP crystal changed, depending on the concentration of Ca and PO_4 solutions used as ionic sources (figure 1). Plate-like crystals grew, when 5mM solutions, which were minimum concentration to form crystals in the present condition, were used. Length of OCP crystal increased from about 13±4 µm to 91±7µm with an increase in concentration of Ca and/or PO_4 solutions. Increase in length was smaller than expected, when the solution concentration was higher than 10mM, because crystal grew on both CMV and dialysis membrane, while they grew only on the CMV when the concentrations were lower than 10mM. The width decreased under a large amount of ionic flow. The drastic change of morphology from rectangle to long ribbon is figured by the length to width (L/W) and width to thickness (W/T) ratios (figure 2) : The L/W ratio changed from 3 to 95, whereas the W/T ratio changed between 32 and 8, as the concentration of Ca and/or PO_4 solutions increased. Thus, longer and narrower ribbon-like crystals grew, when the amount of flux across the membranes was large.

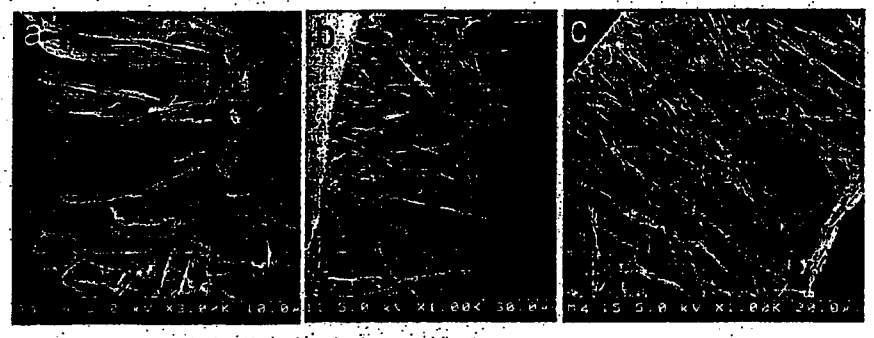

Figure 1. Morphology of OCP crystals grown under various amount of PO_4^{3-} and Ca^{2+} ions' influx. Concentration of Ca and PO_4 solutions used as ionic sources are (a) Ca 5mM, PO_4 5mM ; (b) Ca 5mM, PO_4 30mM ; (c) Ca 10mM, PO_4 30mM.

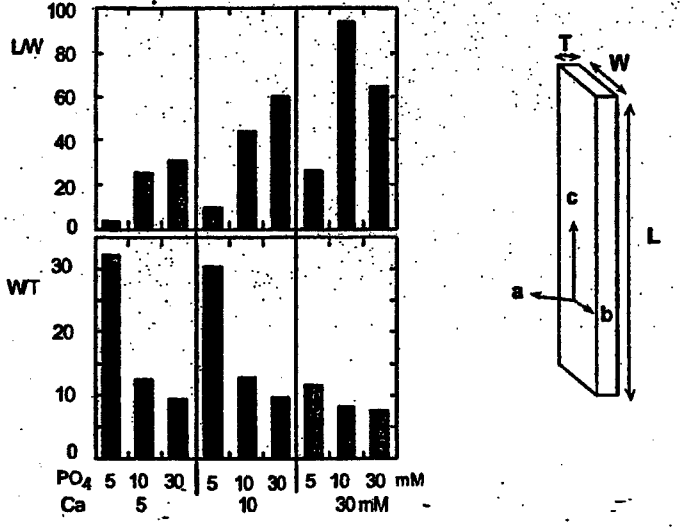

Figure 2. Length to width (L/W) ratio and width to thickness (W/T) ratio of OCP crystals grown under various ion flows [15]. The scheme shows morphology and crystallographic axes of OCP crystal.

Effects of amelogenins

The effect of amelogenins on the crystal morphology was unique (figure 3) : Ribbon-like crystals grew in the absence of organic materials (control) and in gelatin, albumin, PAA gel and agarose gel : In contrast, prism-like and rod-like crystals grew in 10% amelogenins, regardless the type of amelogenins. The XRD indicated these products are OCP with good crystallinity.

Drastic reduction in length and width of OCP crystal was caused by organic materials with different efficiency (figure 3, 4). The length decreased from 90±8μm (control) to 6±2μm (bovine amelogenins) and 3.6±1μm (albumin), and the width from 2±0.5μm (control) to 94±35nm (bovine amelogenins) and 83±17nm (albumin). The inhibitory activity of the bovine amelogenins in length was larger than that of rM179 and rM166. Both rM166 and

rM179 had similar effect on the growth of OCP. The inhibitory activity of amelogenins gels in length and width were larger than that of PAA gel. Whereas, the decrease in thickness caused by amelogenins gels was smaller than that caused by other materials. The inhibitory effect of albumin was the largest.

The degree of crystal size reduction in 10% amelogenins gels was in the order of width, length and thickness. This means that amelogenins suppressed the growth of OCP in the order, b-axis > c-axis > a-axis direction and that the interaction of amelogenins with the crystal face of OCP was in the order of (010) > (001) > (100).

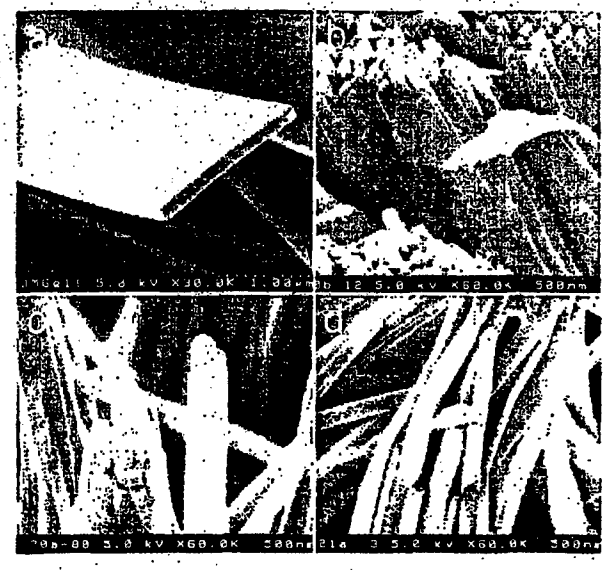

Figure 3. Morphology of crystals grown (a) without additives, in (b) 10% albumin, (c) 10% rM179, and (d) 10% rM166. Note that both rod-like and prism-like crystals were obtained in three types of amelogenins, i.e., bovine amelogenin, rM179 and rM166.

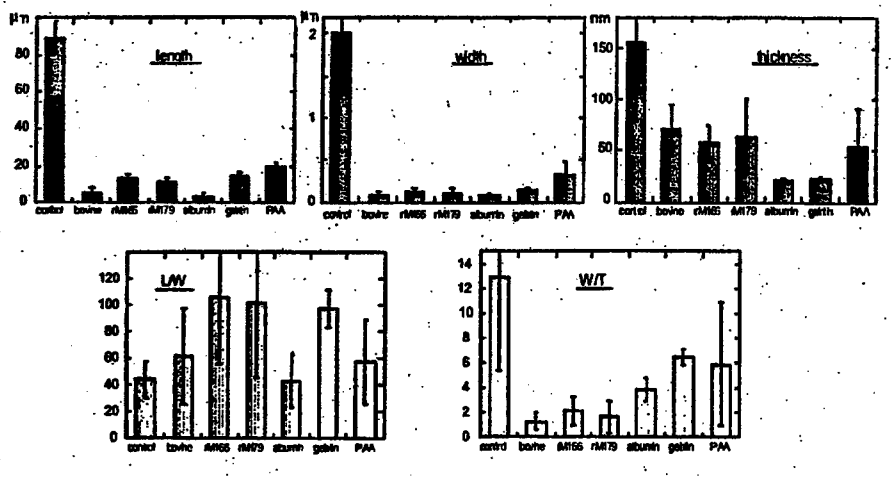

Figure 4. Length (L), width (W), thickness (T), L/W ratio and W/T ratios of OCP crystals grown without proteins and in 10% bovine amelogenins, rM179, rM166, albumin and gelatin.

The L/W and W/T ratios of the crystals (figure 4) show the unique effect of amelogenins on morphology. Bovine amelogenin, rM179 and rM166 resulted crystals with the L/W ratio larger than that of control crystals and with the smallest W/T ratio. In case of rM166 and rM179, the L/W ratio was about 2.5 times larger than and the W/T ratio was about 1/6 times smaller than those of control crystals. Gelatin also caused the elongation as well as rM166 and rM179, but the W/T ratio was 1/2 of the control crystal.

DISCUSSION

The results of our in vitro experiments demonstrate that both ionic flow and amelogenin contributed to form OCP crystals with elongated morphology. Under a larger amount of ionic flow, the lengthwise growth was enhanced, while the growth in the width direction was reduced. OCP crystal grew preferentially in the c-axis direction, while grew less actively in the b-axis direction. The (001) face of OCP crystal was, presumably, the most active face on which ions and molecules of the lattice components were attached and subsequently OCP structure was quickly constructed in the c-axis direction. As a result, the maximum L/W ratio of crystals was about 25 times larger than that of crystal grown under the smallest ionic flow (figure 2). In the absence of organic materials, even when the ionic flow changed, crystal length and width were not reduced less than 13 and 4 μm, respectively. When the ionic flow was further decreased, i.e., when the concentration of the ionic sources were less than 5mM, there was no precipitation on the membrane. Drastic reduction in crystal size was caused by organic materials with different efficiency (figure 4).

Amelogenin gels caused a large decrease in width and small decrease in thickness of OCP crystal. As a result, decrease in W/T ratio caused by 10% amelogenin gels was larger than those caused by other organic materials used (figure 4). The W/T ratio, 1.3, was much smaller than that caused by the change in the amount of ion flow in the absence of amelogenins (figure 2). The effect of amelogenins on crystal morphology was common among bovine amelogenins, rM166 and rM179, regardless of the homogeneity of molecular weight and the existence of the hydrophilic C-terminal. This indicates that the specific interaction of amelogenins with the crystal faces of OCP related to some common factors among these amelogenins. That is hydrophobicity [14,16], because most part of amelogenin molecules are composed with hydrophobic amino acids, except the C-terminal of rM179 [20]. According to the crystal structure analysis [21], OCP has ten H_2O molecules per one unit cell. Amount of H_2O molecules exposed in its crystal face is in the order of (100) > (001) >> (010). Therefore, it is expected that the (100) and (001) faces are hydrophilic, while the (010) face is rather hydrophobic. The ionic arrangement in its crystal face suggests that the (100) and (010) faces are positive, while the (001) face is slightly negative. The amount of the positive charge of the (100) is higher than that of the (001). Amelogenin molecules are hydrophobic and positive at pH6.5, and they assemble into nanospheres with hydrophobic property [8-10]. AFM imaging of the

10%, 30% and 35% amelogenin gels revealed that those nanospheres were basic building block of the amelogenin gels [16,22,23]. On the other hand, PAA gel has the closed-cellular structure with the pore size of a few micron [24]. Due to the cell walls, the mechanical interference of the PAA gel was supposed to be larger than that of the amelogenin gels. Nevertheless, the decrease in length by amelogenin gels was larger than that by PAA gel. This also supports the interaction with amelogenin nanospheres with the (001) face of OCP crystal. The hydrophobic and positive nanospheres would react with the hydrophobic (010) and negative (001) faces rather than with the hydrophilic and positive (100) face. This coincides with the result that the degree of the interaction was in the order of (010) > (001) > (100).

During tooth enamel formation, Ca^{2+} and PO_4^{3-} ions are transported from the layer of ameloblasts into the enamel matrix and the mode of ionic transports change during enamel formation, accompanied with the changes in activity of these ions and the Ca/ PO_4 ratio [3,4]. Recent studies have postulated that amelogenin nanospheres play active roles in the elongation of enamel crystals [9,13]. In the dual membrane system, it was demonstrated that the L/W ratio was increased about 25times by increasing the amount of the ionic flow and it was increased in 10% amelogenin gels 1.5-2.5 times larger than that of control crystals [14-16], thereby, indicating that the ionic flow and amelogenin gels played a key role in the lengthwise growth of OCP crystal. In a gelatin gel system, 1-2% amelogenins effectively elongated OCP crystals, which have the L/W ratio 3-5times larger than that of control crystals [25]. The width and thickness of human enamel crystals in the early stage are 15nm and 1.5nm, respectively, and those in maturation stage are 68nm and 26nm, respectively [2]. Since enamel crystals are long, not straight and fragile, it is difficult to measure the length. It is reported that enamel crystals extend over an entire layer from the dentino-enamel junction to Tomes' processes of ameloblasts [26] and they are at least 100μm [27], which maybe the longest reported value. When the length and the width were 100μm and 15nm, respectively, the L/W is 6667. When the length and width were 100μm and 68nm, respectively, the L/W is 1470. The extremely large L/W ratio of enamel crystals might still require other factors/mechanism to be involved, which work to reduce the width (i.e., the growth in the b-axis direction) and to increase the length (i.e., the growth in the c-axis direction) in combination with amelogenins and ionic flow.

CONCLUSIONS

The increase in the amount of calcium and phosphate ionic flow enhanced OCP crystal to grow longer and narrower. Organic materials reduced crystal size. The effect of amelogenins on the morphology of OCP was unique, when it was compared with albumin, gelatin, PAA, and agarose. Only 10% amelogenin gels changed the morphology from ribbon-like to prism-like with large L/W ratio and small W/T ratio. Amount of water molecules and electric charge of the (100), (010) and (001) face of OCP crystal reflected the degree of interaction with amelogenins. The present study supported the view that the ionic flow and amelogenin nanospheres play key roles in controlling the lengthwise and oriented growth of enamel crystal.

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance of Mr. Narbeh Gharakhain in protein expression and purification. This work was supported by NIH-NIDCR research grant DE12350 and DE13414 (J.M.O.) and by the Miyata Grant from Asahi University (M.I.).

REFERENCES

1. M.U. Nylen, E.D. Eanes and K.A. Omnel. J. Cell Biol. 18, 109 (1963).
2. B. Kerebel, G. Daculsi and L.M. Kerebel. J. Dent. Res. 58B, 844 (1979).
3. T.Kawamoto and M.Shimizu. Calcif. Tissue Int. 46, 406 (1990).
4. T.Kawamoto and M.Shimizu. Jpn. J. Oral Biol. 36, 365 (1994).
5. J.F. Eastoe. J Dent. Res. 58B, 753 (1979).
6. J.D. Termine, A.B. Belcourt, P.J. Christener, M.K. Conn and M.U. Nylen. J Biol. Chem. 255, 9760 (1980).
7. T. P. Hopp and K. R. Woods, Proc. Natl. Acad. Sci. USA 78, 3824 (1981).
8. J. Moradian-Oldak, J.P. Simmer, F.C. Lau, P.F. Sarte, H.C. Slavkin and A.G. Fincham, Biopolymers 34, 1339 (1994).
9. A.G. Fincham, J. Moradian-Oldak, T.G.H. Diekwisch, D.M. Layarun, J.T. Wright, P.Bringas and H.C. Slavkin, J. Struct. Biol 115, 50 (1995).

10. J. Moradian-Oldak, W. Leung and A.G. Fincham, J Struct Biol. 122, 320 (1998).
11. G. Nikifolk and N.S. Simmons, J. Dent. Res. 44, 1119 (1965).
12. M. Iijima, "Formation of Octacalcium Phosphate in vitro", *Monographs in Oral SciVol.18. / Octacalcium Phosphate*, ed. L.C. Chow and E.D. Eanes (Karger AG, 2001) pp17-49.
13. J. Moradian-Oldak, Matrix Biology 20, 293 (2001).
14. M. Iijima, Y. Moriwaki, T. Takagi and J. Moradian-Oldak, J. Crystal Growth 222, 615 (2001)
15. M. Iijima, K. Hayashi and Y. Moriwaki, J. Crystal Growth 234, 539 (2002).
16. M. Iijima, Y. Moriwaki, H.B. Wen, A.G. Fincham and J. Moradian-Oldak, J. Dent. Res. 81, 69 (2002).
17. T. Takagi, M. Suzuki, T. Baba, K. Minegishi and S. Sasaki, Biochem. Biophysics. Res. Comm. 121, 592 (1984).
18. H. Shimokawa, Y. Ogata, S. Sasaki, M.E. Sobel, C.I. McQuillan, J.D. Terminc and M.F. Young, Adv. Dent. Res. 1, 293 (1987).
19. J.P. Simmer, E.C. Lau, C.C. Hu, P. Bringas , V. Santos, T. Aoba, M. Lacey, D. Nelson, M. Zeichner-David, M.L. Snead, H.C. Slavkin and A.G. Fincham Calcif. Tissue Int. 54, 312 (1994).
20. T.P. Hopp and K.R. Woods, Proc. Natl. Acad. Sci. USA 78, 3824 (1981).
21. W.E. Brown, J.P. Smith, J.R. Lehr and A.W. Frazier, Nature 196, 1048 (1962).
22. H.B. Wen, J. Moradian-Oldak, W. Leung, P.Jr. Bringas and A.G. Fincham, J. Struct. Biol. 126, 42 (1999).
23. H.B. Wen, A.G. Fincham and J. Moradian-Oldak, Matrix. Biol. 20, 387 (2001).
24. R. Ruchel and M.D. Brager, Anal. Biochem. 68, 415 (1975).
25. H.B. Wen, J. Moradian-Oldak and A.G. Fincham, J. Dent. Res. 79, 1902 (2000).
26. T.G.H. Diekkwisch, B.J. Berman, S. Gentner and H.C. Clavkin, Cell Tissue Res. 279, 149 (1995).
27. G. Daculsi, L.M. Kerebel and D. Mitre, "Enamel crystals : size, shape, and length and growing process ; high resolution TEM and biochemical study." *Tooth Enamel IV*, ed. R.W. Fearnhead and S. Suga, (Elsevier, 1984) pp.14-18.